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Copper and ocean acidification interact to lower maternal investment, but have little effect on adult physiology of the Sydney rock oyster *Saccostrea glomerata*

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ABSTRACT

It remains unknown how molluscs will respond to oceans which are increasingly predicted to be warmer, more acidic, and heavily polluted. Ocean acidification and trace metals will likely interact to increase the energy demands of marine organisms, especially oysters. This study tested the interactive effect of exposure to elevated pCO_2 and copper on the energetic demands of the Sydney rock oyster (*Saccostrea glomerata*) during reproductive conditioning and determined whether there were any positive or negative effects on their offspring. Oysters were exposed to elevated pCO_2 (1000 μatm) and elevated copper (Cu 50 μg L^{-1} [0.787 μM]) in an orthogonal design for eight weeks during reproductive conditioning. After eight weeks, energetic demands on oysters were measured including standard metabolic rate (SMR), nitrogen excretion, molar oxygen to nitrogen (O:N) ratio, and pH_e of adult oysters as well as the size and total lipid content of their eggs. To determine egg viability, the gametes were collected and fertilised from adult oysters, the percentage of embryos that had reached the trochophore stage after 24 h was recorded. Elevated pCO_2 caused a lower extracellular pH and there was a greater O:N ratio in adult oysters exposed to copper. While the two stressors did not interact to cause significant effects on adult physiology, they did interact to reduce the size and lipid content of eggs indicating that energy demand on adult oysters was greater when both elevated pCO_2 and copper were combined. Despite the lower energy, there were no negative effects on early embryonic development. In conclusion, elevated pCO_2 can interact with metals and cause greater energetic demands on oysters; in response oysters may lower maternal investment to offspring.

1. Introduction

The legacy of poor environmental management lingers in estuaries around the world. Anthropogenic rubbish, persistent organic compounds, nutrients and trace metals pollute estuaries adjacent to urban and industrial areas (Nriagu, 1990; Ridgway and Shimmield, 2002; Halpern et al., 2008). Despite their degraded nature, polluted estuaries support a diversity of marine organisms including bivalve oysters. Oysters are integral to functioning estuaries because they filter the water column, cycle nutrients and provide biogenic habitat, but these traits also make oysters vulnerable to toxic pollutants like trace metals (Connell, 1990). Exposure to trace metals can affect cellular function, damage DNA, and ultimately lead to cellular death because oysters exposed to trace metals need to expend energy on protein synthesis to repair and detoxify cells (Cherkasov et al., 2006; Sokolova and Lannig, 2008).

As oceans warm and acidify over the next century (Collins et al., 2013) oysters and other bivalves will be particularly vulnerable (Gazeau et al., 2013). Several studies have shown that ocean acidification alone will alter the physiology of oysters and restrict their capacity to grow and reproduce (Pörtner, 2001; Pörtner et al., 2004; Fabry, 2008; Doney et al., 2009; Parker et al., 2013). Other stressors like low-salinity, air exposure or trace metals have been shown to exacerbate the effects of elevated CO_2 (Ivanina and Sokolova, 2015; Parker et al., 2017a, b; Scanes et al., 2017).

Ivanina and Sokolova (2015) found that elevated partial pressure of CO_2 (pCO_2) and trace metals acted indirectly on a range of metabolic and physiological processes in oysters but it is difficult to identify the mechanisms that underpin such interactions. Lewis et al. (2016) found copper and CO_2 interacted synergistically to reduce extracellular pH (pH_e) in the mussel *Mytilus edulis*. The mussel respired less when exposed to the trace metal copper. Respiring less had no effect on pH_e

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when copper was a sole stressor, but when copper was combined with elevated pCO_2 the authors found that the mussel could not ventilate the excess CO_2 which caused internal pCO_2 to increase, and as a result, synergistically lower pH_e (Lewis et al., 2016). Trace metals and elevated pCO_2 also interacted to affect the proteome expression in bivalves (Götze et al., 2014). When exposed to elevated copper and CO_2, the clam *Mercenaria mercenaria* increased its energy use to synthesise new proteins and repair damaged cells (Götze et al., 2014).

Environmental stress can increase the cost of homeostasis and place strong demands on the energy budget of marine invertebrates like oysters (Sokolova, 2013). Investing extra energy into homeostasis often comes at the expense of other functions such as somatic growth, digestion and reproduction. This may begin to explain why trace metals and ocean acidification interact to cause a diverse range of effects on marine bivalves (Sokolova, 2013; Ivanina and Sokolova, 2015). However, our understanding of how trace metals and ocean acidification interact is based on short term, single generation studies, an approach that neglects the real-world scenario where organisms respond over multiple generations.

Exposure to stress can cause carryover effects (both positive and negative) across generations to offspring. The environment that adults experience while they are developing gametes (called reproductive conditioning) is known to significantly influence the response of their offspring. When Parker et al. (2012) exposed oysters to elevated CO_2 throughout reproductive conditioning, it was found that the oyster's offspring were larger, and more resilient to ocean acidification as both larvae and adults (Parker et al., 2015). Parents can influence their offspring by allocating extra energy and resources (called maternal investment). In some cases, mothers that are stressed during conditioning may produce larger eggs, so larvae have more energy (Marshall and Uller, 2007). Alternatively, stress from sources like metals can cause parents to invest less energy resulting in smaller eggs which lead to smaller juveniles (Marshall and Uller, 2007; Alquezar et al., 2006). For example, parents of marine fish have been shown to produce smaller eggs when exposed to the trace metal copper (Alquezar et al., 2006). While stressors like trace metals or ocean acidification can influence offspring when parents are exposed during conditioning, no study has investigated how these stressors may interact to affect oysters and have positive or negative carryover effects on their offspring.

Oysters are ubiquitous on the Australian coastline and often inhabit polluted locations adjacent to urban and industrial areas. The Sydney rock oyster (*Saccostrea glomerata*) is prevalent on the east coast of Australia and inhabits urbanised estuaries such as Sydney Harbour which is polluted with copper (Mayer-Pinto et al., 2015; Scanes et al., 2016). Copper is a common estuarine pollutant in Australia and worldwide (Nriagu, 1990; Mayer-Pinto et al., 2015). In addition to its prevalence, copper is also known to cause significant biological harm to both aquatic and terrestrial animals at the concentrations often found estuaries (Ridgway and Shimmield, 2002). Sydney rock oysters are recognised as vital to functioning ecosystems on the Australian coast (Cole et al., 2007). This study tested the interactive effect of chronic exposure to elevated pCO_2 and the trace metal copper on the energetic demands of the Sydney rock oyster *S. glomerata*, and determined any carryover effects to offspring. It was predicted that elevated pCO_2 combined with copper would interact to affect energetic demands and adult physiology as well as affect gamete quality and survivorship of offspring.

2. Methods

S. glomerata were exposed in the laboratory to elevated pCO_2 predicted for the end of this century (Collins et al., 2013) and aqueous copper (determined by Edge et al., [2015] in previous experiments to be stressful for *S. glomerata*). Oysters were exposed in an orthogonal combination for eight weeks during reproductive conditioning. To determine the energetic state of adults, their physiology was measured including; standard metabolic rate (SMR), nitrogen excretion, molar O:N ratio, and extracellular pH (pH_e). To determine the impact on maternal energy investment into eggs, reproductive variables such as egg size and lipid content were measured (Yasumasu et al., 1984; Gallager et al., 1986; Moran and McAlister, 2009). To determine the effect of parental exposure on offspring viability, after eight weeks of exposure, gametes were collected, fertilised and larval viability assessed.

2.1. Collection of oysters

No ethics approval was required by New South Wales (NSW) or Australian law for this experiment. Adult *S. glomerata* were collected from a commercial oyster grower in Port Stephens NSW, Australia. Oysters were approximately two years old and had been maintained on oyster leases within Cromarty Bay, Port Stephens (32°43′28.83″S, 152° 3′51.14″E). Oysters were collected in the Austral winter (June) 2016 and were then cleaned of all fouling organisms and placed into two 750 L tanks filled with nominal 1 µm filtered seawater (FSW hereafter) at 17 °C, which was incrementally raised to 22 °C over three days. Oysters were then kept in these tanks for another 5 days (8 total) to acclimate to laboratory conditions and recover from handling. Thirty individuals were opened immediately following collection to determine gonadal condition. Macroscopic observation of the gonad surface and microscopic (Leica 400x) observation of gonad smears confirmed the oysters were in poor reproductive condition (regressive – early ripening, after Dinamani, 1974).

2.2. Experimental treatments for adult parental exposure

Adult oysters were conditioned in experimental treatments for eight weeks during which they were exposed to one of four orthogonal nominal experimental treatments: control (ambient pCO_2, 400 µatm; and no copper, Cu 0 µg L^{-1}[0 µM]), elevated pCO_2 and no copper (1000 µatm; Cu 0 µg L^{-1} [0 µM]), ambient pCO_2 and elevated copper (400 µatm, Cu 50 µg L^{-1} [0.787 µM]) or elevated pCO_2 and elevated copper (1000 µatm; Cu 50 µg L^{-1} [0.787 µM]). Each treatment was replicated three times in independent 120 L polyethylene tanks (total 12 tanks; Fig. 1). Within each tank there were 15 oysters suspended in a mesh bag (20 mm aperture).

2.3. CO_2 monitoring

The two pCO_2 levels used in this study (390 µatm, 1000 µatm) were based on the multi-model average projection by the IPCC for 2100 (Collins et al., 2013). This equated to a mean ambient pH_{NBS} of (8.19 ± 0.02) and a mean pH_{NBS} at elevated CO_2 levels of (7.84 ± 0.0035; Table 1). Gaseous CO_2 was added to tanks using a negative feedback system as described in detail by (Parker et al., 2012). Briefly, the elevated pCO_2 level was maintained using a pH negative feedback system (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia; accuracy ± 0.01 pH units). To determine the pH level corresponding to pCO_2 levels, total alkalinity (TA) was quantified at each water change using triplicate Gran-titration (Gran, 1952). The pH_{NBS}, total alkalinity, and salinity of each thank were also measured at each water change (Table 1). These measurements were then entered into a CO_2 system calculation program (CO_2SYS; Lewis et al., 1998), using the dissociation constants of (Mehrbach et al., 1973) to calculate the desired pH values corresponding with pCO_2 levels. The pH values of each tank were monitored daily, and the pH electrode of each controlling system was checked daily against another calibrated pH probe (NBS buffers, WTW 3400*i*).

Copper treatments have been pooled within their respective pCO_2 treatments. Total alkalinity (TA), Temperature (°C), pH_{NBS}, and salinity were measured every water change. All other variables were calculated using a CO_2 system calculation program (Lewis et al., 1998), using the

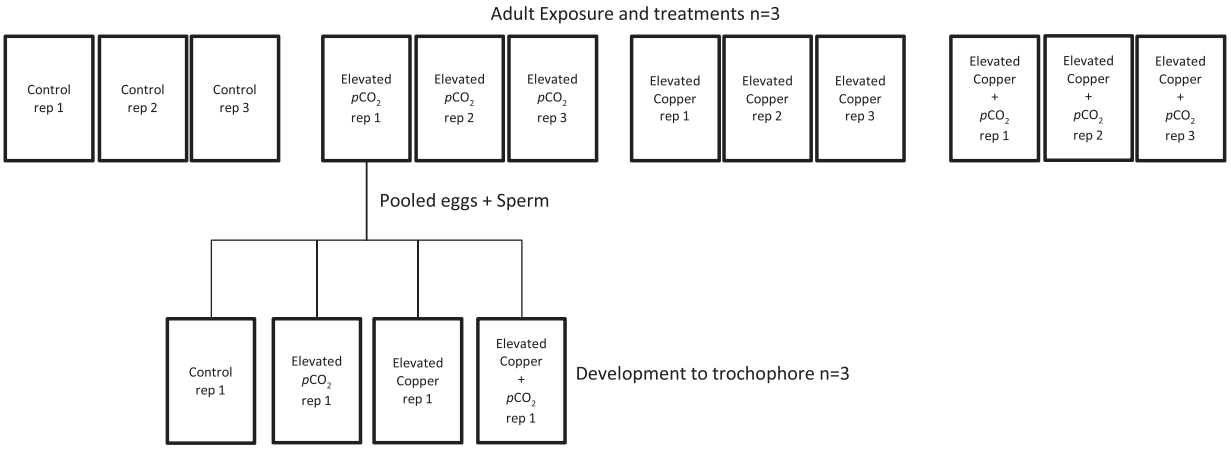

Fig. 1. Diagram of experimental design for development to trochophore. The first row of tanks represents the adult exposure treatment Pooled eggs and sperm from three oysters in each replicate tank were then stocked into four 120 mL jars set at the four levels of experimental treatment. This was repeated for each replicate tank of adult oysters. Experimental treatments for both adult exposure and then development to trochophore were; control (ambient pCO_2, 400 µatm; and no copper, Cu 0 µg L^{-1}), elevated pCO_2 and no copper (1000 µatm; Cu 0 µg L^{-1}), ambient pCO_2 and elevated copper (400 µatm, Cu 50 µg L^{-1}) and elevated pCO_2 and elevated copper (1000 µatm; Cu 50 µg L^{-1}).

Table 1
Mean (± SE) physiochemical variables of seawater, from each pCO_2 treatment level over the eight-week experimental exposure period (n = 6).

Nominal pCO_2 treatment	400 µatm	1000 µatm
TA (µEqKg^{-1})	2353.09 ± 20.15	2353.09 ± 20.15
Temperature (°C)	22 ± 0.5	22 ± 0.5
pH_{NBS}	8.12 (± 0.005)	7.83 (± 0.02)
Salinity (ppm)	32 ± 0.5	32 ± 0.5
pCO_2(µatm)	395 ± 11.32	1000 ± 1.4
DIC	2072 ± 9.6	2237 ± 15.8
$\Omega_{calcite}$	4.92 ± 0.18	2.49 ± 0.04
$\Omega_{aragonite}$	3.21 ± 0.15	1.62 ± 0.03

dissociation constants of (Mehrbach et al., 1973).

2.4. Adult parental exposure to copper

Analytical grade copper chloride ($CuCl_2$; Sigma-Aldrich Co.) was added as a contracted stock solution to give a final nominal concentration of [Cu^{2+}] 50 µg L^{-1} (0.787 µM) in each 120 L tank at each water change, prior to the oysters being returned to the tank. The concentration of [Cu^{2+}] 50 µg L^{-1} (0.787 µM) was chosen because it is environmentally relevant (Birch and Taylor, 1999) and at this concentration [Cu^{2+}] is known to cause significant stress to *S. glomerata* through water borne exposure in the forms of lysosomal destabilisation and lipid peroxidation (Edge et al., 2015). Water samples were taken once monthly throughout the experimental exposure from each tank to analyse for Cu concentrations. To sample for copper analysis of seawater, each tank containing oysters was stirred and a 10 mL syringe was used to extract a water sample, which was then filtered to 0.2 µm and placed into a new analytical vial. Trace metal analysis grade nitric acid (HNO_3 0.1 mL; TraceSELECT® Sigma-Aldrich Co.) was then added to each vial, which were stored in a refrigerator for later analysis.

To analyse oyster tissue for total copper content, the entire internal flesh of one individual oyster from each replicate 120 L tank was taken following the eight weeks of experimental exposure. The whole internal contents of the animal were freeze dried for 24 h and then homogenised using a ceramic ball mill (Retsch, Gmbh-400 mm, Germany). Ground tissue was then digested in nitric acid using a high temperature microwave digester following manufacturer guidelines (Milestone START D; PRO 24 high throughput rotors; Italy). To prepare tissue for analysis a single batch of digestions was conducted. Briefly, 0.1 g of freeze dried tissue was digested in 10 mL of HNO_3 (TraceSELECT® Sigma-Aldrich Co.) for 18 h at room temperature and then 18 min at 200 °C. Copper concentrations in seawater and tissue digest samples were determined using Inductively-Coupled Plasma Mass Spectroscopy (Perkin Elmer Nexion 300x Inductively Coupled Plasma Mass Spectrometer; ICPMS), on Helium Collision KED (Kinetic Energy Dispersion) mode. Copper mass was analysed against a standard series covering the expected range of the samples. Prior to analysis, samples were diluted by a factor of 20:1 to reduce the effect of high saline matrix, and spiked with an internal standard (Rhodium). For quality assurance, oyster reference material (NIST 1566b, Gaithersburg, USA) and a blank (HNO_3 only) were digested and analysed alongside the oyster tissue digest samples. Analysis of certified oyster reference material (NIST 1566b) indicated a 98.2% recovery of Cu. Concentrations of Cu in oyster tissue were standardised to the oyster dry weight (g^{-1} dry wt.).

2.5. Cleaning and feeding

Twice a week, each tank received a complete water change. A second set of polyethylene tanks were filled with FSW and equilibrated to the temperature and if appropriate, the pCO_2 level/ Cu^{2+} level of corresponding tanks already housing oysters. Oysters were then transferred to a clean tank (of the same treatment variables), ensuring they were out of the water briefly. Tanks were completely drained and then scrubbed clean using Virkon S solution (Antec Corp, North Bend, WA, USA). Oysters were fed each day an algal mixture consisting of 50% *Chaetoceros muelleri* and 50% *Tisochrysis lutea* at a concentration equivalent to 2×10^9 *T. lutea* cells oyster^{-1} d^{-1}. During water changes, tanks were inspected for dead oysters. Deaths were recorded, and oysters were replaced to maintain stocking densities, however replacement oysters were marked and not used for any measurements.

2.6. Physiological measurements of adult oysters

2.6.1. Standard metabolic rate and condition index

After eight weeks of experimental exposure, standard metabolic rate (SMR) of adult oysters was measured using a closed respiratory system as described in detail by (Parker et al., 2012). In each treatment, two individuals were randomly selected from each replicate tank for measurements. Briefly, Oysters were placed in individual 500 mL airtight chambers filled with FSW set to the corresponding pCO_2 or copper level of that treatment, and the change in oxygen was measured over approximately two hours using a fibre-optic O_2 probe (PreSens dipping probe DP-PSt3, AS1 Ltd, Regensburg, Germany). Following the

measurements, oysters were removed from the chambers, opened, and tissue was separated from their shell. Both tissue and shells were dried in an oven at 70 °C for 72 h then weighed using an electronic balance (± 0.001 g). SMR was then calculated for each individual using Eq. (1). Condition index was calculated using Eq. (2).

$$\mathrm{SMR} = \frac{[Vol \times \Delta C_W O_2]}{\Delta t \times W} \quad (1)$$

Where: SMR is oxygen consumption normalised to 1 g of dry tissue mass (mg O_2 g^{-1} dry tissue mass h^{-1}), *Vol* is the volume of the respiratory chamber minus the volume of the oyster (L), $\Delta C_W O_2$ is the change in water oxygen concentration measured (mg O_2 L^{-1}), Δt is the measuring time (h), *W* is the dry tissue mass (g) (Parker et al., 2012).

$$\mathrm{CI} = \left(\frac{W_b}{W_s}\right) \times 100 \quad (2)$$

Where: CI is the condition index, W_b is the dry tissue mass (g), W_s is the dry shell mass (g).

2.6.2. Oxygen consumption and nitrogen excretion

To determine the molar equivalent ratio of oxygen consumption to nitrogen excretion, measurements of oxygen (O_2) and ammonia (NH_3) concentrations (μg L^{-1}) were taken at the beginning and end of SMR incubations, and then standardised to time and dry tissue mass. Oxygen was measured using the fibre-optic O_2 probe (PreSens dipping probe DP-PSt3, AS1 Ltd, Regensburg, Germany) as per SMR measurements, at the beginning and end of incubations. Nitrogen was measured as NH_3 by taking a 5 mL homogenous seawater sample from each chamber at the beginning and end of incubations. Samples were immediately filtered to 0.2 μm and then frozen at −20 °C until analysis. Samples for ammonia analysis were pre-digested using persulfate. Concentrations of ammonia were then determined using flow-injection spectrometry (Lachat Quickchem 8500) according to standard methods (American Public Health Association (APHA, 1994). Ammonia excretion and oxygen consumption were then standardised to incubation time and dry tissue mass using Eq. (3) which was derived from Bayne (1999). The molar ratio of oxygen consumed to nitrogen excreted was then calculated by dividing the molar equivalent of O_2 by NH_3.

$$V_{\mathrm{stand}}\,\mathrm{h}_{-1} = \left(\frac{Vol \times \Delta V_{\mathrm{meas}}}{\Delta t}\right) \times W^{-1} \quad (3)$$

Where; V_{stand} is calculated measured variable (O_2 or NH_3) normalised to g^{-1} of dry tissue mass, *Vol* is the volume of the respiratory chamber minus the volume of the organism (L), ΔV_{meas} is the change in concentration of the measured variable (μmol L^{-1}), Δt is the whole incubation time (h), and *W* is the dry tissue mass (g)

2.6.3. Extra-cellular pH

To determine the effects of experimental treatments on oysters' extra cellular pH (pH_e) following eight weeks exposure, two oysters were randomly taken from each replicate tank. Oysters were then immediately opened without rupturing the pericardial cavity. Haemolymph samples were drawn from the interstitial fluid filling the pericardial cavity chamber of an opened oyster using a sealed 1 mL needled syringe. A 0.2 mL sample was drawn carefully to avoid aeration of the haemolymph. The sample was then immediately transferred to an Eppendorf tube where pH_e of the sample was measured at 22 °C using a micro pH probe (Metrohm 827 biotrode) calibrated prior to use following manufactures guidelines with NBS standards at the experimental temperature of 22 °C.

2.7. Eggs size, lipid content, and embryonic development

2.7.1. Spawning

To obtain gametes, three female and three male oysters from each replicate tank were collected following 8 weeks of experimental exposure. Each individual oyster was opened, the gonad was scored with a scalpel blade, and the gametes were rinsed out through a 150 μm screen (to catch debris) using a wash bottle containing FSW into a 500 mL beaker. A small sample of gametes from each oyster was observed by microscopy (Leica 400x) to determine sex. The density of eggs collected from each female oyster was determined using a Sedgewick-rafter slide and light microscopy (Leica 400x). Three females from the same replicate tank each contributed 50,000 eggs which were then pooled together in a single beaker to give an equal representation for three females from each replicate tank. Pooled eggs were then allowed to stand for 30 min. to "water harden" and gain their spherical shape (Parker et al., 2017a). The total number and density of eggs in the pooled beaker was then also determined using a Sedgewick-rafter slide and light microscopy (Leica 400x). The sperm from each male oyster was kept separate and cool until fertilisation.

2.7.2. Egg size

To determine whether egg size differed among treatments, pooled eggs were homogenised, and a 2 mL subsample (approx. 30 000–50 000 eggs) was taken from each of beaker containing pooled eggs from three females that represented a replicate tank. Twenty individual eggs within one subsample were measured (μm) using a Sedgewick-rafter slide and ocular micrometer under a light microscope (Leica 400x).

2.7.3. Lipid analysis

To determine the lipid content of eggs, a second 2 mL subsample (approx. 30 000–50 000 eggs) of eggs was collected from each beaker of pooled eggs (three females), placed into a centrifuge tube and then centrifuged at 3000 RPM for 2 min. The excess water was then drained off and eggs were stored at −80 °C until lipid analysis. Egg samples were quantitatively extracted overnight using a modified Bligh and Dyer (1959) one-phase methanol-chloroform-water extraction (2:1:0.8 v/v/v). The phases were separated by the addition of chloroform-water (final solvent ratio, 1:1:0.9 v/v/v methanol-chloroform-water). The total solvent extract (TSE) was concentrated using rotary evaporation at 40 °C. Abundances of individual lipid classes including acylglycerides were determined using an Iatroscan (MK VI TH10 TLC-FID) analyser following the methods described previously in Phleger et al. (1997). Percentage error of lipid analysis from the calibration curve was a maximum of 3.9%. Total lipid content and each identified lipid class were standardised to μg egg^{-1}.

2.7.4. Embryonic development

To determine the effects of parental exposure to elevated pCO_2 and copper on fertilisation and early development, an orthogonal experiment was designed. Eggs were fertilised and development was allowed to proceed under the same treatments as used for the exposure of adult oysters. Pooled eggs collected from three adult female oysters from each replicate tank were stocked into four 120 mL jars containing FSW set at the four experimental treatments described previously (Fig. 1). This gave a total of 48 jars, one set at each treatment level for each replicate tank (Fig. 1).

Jars were stocked at a density of 30 eggs mL^{-1}. The pCO_2 of experimental jars was manipulated via the constant delivery of premixed CO_2 in air at 1000 μatm (BOC gas co.) to give a pH of 7.84 (checked by calibrated pH probe; WTW 3400*i*). Three individual tanks of premixed gas supplied each of three replicate jars per treatment. Treatments that were exposed to ambient pCO_2 were continuously bubbled with air at the same rate as the elevated pCO_2 jars. Copper concentrations in jars were manipulated by adding a concentrated stock solution of analytical grade $CuCl_2$ (Sigma-Aldrich Co.) to give a nominal concentration of 50 μg L^{-1} (0.787 μM). Sperm collected from the three males per replicate tank were checked to ensure motility, and then pooled in equal quantities. Sperm concentrations were determined using light microscopy (Leica 400x) and a haemocytometer. Sperm was then added to jars containing eggs at a concentration of 5×10^7 sperm mL^{-1} and

homogenised. Following 24 h, the entire contents of the jar was poured over a 20 µm screen. The contents were then resuspended in 1 mL of FSW and transferred to a Sedgewick-rafter slide and observed under a light microscope (Lieca 400x). The ratio of unfertilised eggs to embryos among the first thirty examples observed was recorded, and the stage to which development had occurred was noted. The trochophore stage was identified as ciliated and motile (Morton et al., 1998), this was then expressed as percentage trochophores at 24 h per replicate jar. D-veliger larvae were not observed in jars after 24 h.

2.8. Data analysis

Data from metabolic rate (n = 2), condition index (n = 2), and egg size measurements (n = 20) were analysed using a three factor Analysis of Variance (ANOVA), where "copper exposure" was the first factor, fixed and orthogonal (2 levels; ambient copper and [Cu^{2+}] 50 µg L^{-1}), "pCO_2" exposure was the second factor fixed and orthogonal (2 levels; ambient pCO_2 and elevated pCO_2; 1000 µatm), and "tank" (3 levels; replicate tanks) was the third factor that was random and nested in both "copper exposure" and "pCO_2". The total lipid of eggs (n = 3), acylglyceride content of eggs (n = 3), pH_e (n = 3), deaths (n = 3) and tissue Cu content (n = 3), were analysed using a 2 factor ANOVA where "copper exposure" was the first factor, fixed and orthogonal (2 levels; ambient copper and [Cu^{2+}] 50 µg L^{-1}), "pCO_2" exposure was the second factor fixed and orthogonal (2 levels; ambient pCO_2 and elevated pCO_2; 1000 µatm). Data from embryonic development measurements (n = 3) were analysed using a four factor fully orthogonal ANOVA, "Parental copper exposure" (fixed, orthogonal, 2 levels; ambient copper and [Cu^{2+}] 50 µg L^{-1}) was the first factor, "Parental elevated pCO_2 exposure" (fixed, orthogonal, 2 levels; ambient pCO_2 and elevated pCO_2; 1000 µatm) the second factor, "embryonic copper exposure" (fixed, orthogonal, 2 levels; ambient copper and [Cu^{2+}] 50 µg L^{-1}) the third factor, "embryonic elevated pCO_2 exposure" (fixed, orthogonal, 2 levels; ambient pCO_2 and elevated pCO_2; 1000 µatm) the forth factor. To determine the source of variance in all analysis SNK tests were used *post hoc* on significant factors or interaction of interest (Sokal and Rohlf, 1995). Prior to analyses, the data were tested for homogeneity of variances with Cochran's C test ($\alpha < 0.05$), which confirmed all data satisfied the assumption of homogeneity of variances without the need for transformation. All data were analysed using GMAV-5 for Windows software (Underwood et al., 2002).

3. Results

3.1. Adult physiology and copper content

Elevated pCO_2, but not copper decreased extra-cellular pH of oysters (pH_e) (ANOVA $F_{1,\ 8} = 62.5$; $P < 0.001$; Fig. 2A). There was no significant effect of elevated pCO_2 or copper on the SMR, mean condition index or nitrogen excretion of adult oysters (Fig. 2B and C). At both elevated copper and pCO_2 there was a trend for the SMR of oysters to decrease, being lowest in the combined elevated copper + pCO_2 treatment (Fig. 2C). Oysters showed a non-significant trend to excrete less nitrogen when either copper or pCO_2 were elevated (Fig. 2B). There was a significantly greater ratio of oxygen used to nitrogen excreted (O:N) of oysters when copper was elevated (ANOVA $F_{1,4} = 14.53$; $P < 0.01$; Fig. 2D). There were no significant effects of any treatments on the deaths of oysters (ANOVA $P > 0.3$).

Analysis of the copper content of freeze dried oyster tissue from each treatment after eight weeks of exposure found that there was a mean (± SE; n = 3) concentration of 103 (± 15) µg g^{-1} (1.62 ± 0.23 µmol g^{-1}) in the control treatment, 71 (± 13) µg g^{-1} (1.12 ± 0.2 µmol g^{-1}) in the elevated pCO_2 treatment, 630 (± 63) µg g^{-1} (9.91 ± 0.99 µmol g^{-1}) in the copper treatment and 596 (± 103) µg g^{-1} (9.38 ± 1.62 µmol g^{-1}) in the elevated pCO_2 and copper treatment. There was a significant effect (ANOVA $F_{1,\ 8} = 70.22$; $P < 0.001$) of copper but no effect ($P > 0.4$) of pCO_2 treatment on the copper content of the oyster tissue. Copper concentrations in seawater of copper exposed tanks were approximately 40 µg L^{-1} (0.63 µM) rather than the nominal 50 µg L^{-1} (0.787 µM) that was added (Table 2).

3.2. Egg size and lipid content

Eggs were on average 3 µm (7%) smaller in size from adults exposed to combined elevated copper and pCO_2 (pCO_2 x copper interaction ANOVA $F_{1,8} = 13.61$; $P = 0.006$; Fig. 3A). The total lipid content was also lower in eggs from an elevated copper treatment (ANOVA $F_{1,8} = 6.68$; $P = 0.032$; Fig. 3B). The lowest lipid content of eggs was from the combined elevated copper and pCO_2 which was approximately 50% less than the other three treatments (Fig. 3B). There was also a significant effect of copper exposure on the total acylglyceride (triglyceride, diglyceride, monoglyceride) content of eggs (ANOVA $F_{1,8} = 5.27$; $P = 0.05$; Fig. 3C). Eggs obtained from copper exposed adult oysters had lower total levels of acylglycerides (Fig. 3C).

3.3. Embryonic development

There were less trochophores when embryos were exposed to elevated pCO_2 during development (Embryo CO_2 ANOVA $F_{1,32,} = 10.32$, $P = 0.003$; Fig. 4A). There were, however, more trochophores from parents exposed to the combined elevated pCO_2 and copper treatment when measured at 24 h (Parental copper x Parental pCO_2; ANOVA $F_{1,32} = 20.31$, $P = 0.0001$; Fig. 4B).

4. Discussion

4.1. Adult responses

Exposure to elevated pCO_2 as a sole stressor significantly reduced the pH_e of adult *S. glomerata*. This finding is consistent with most studies that elevated pCO_2 impacts the acid-base balance of oysters (Lannig et al., 2010; Parker et al., 2012; Scanes et al., 2017), and other bivalves (Michaelidis et al., 2005; Schalkhausser et al., 2013; Scanes et al., 2014). Extended periods of lowered pH_e are known to cause a significant reduction in protein synthesis (Kwast and Hand, 1996; Reid et al., 1997), which can ultimately lead to decreased somatic growth (Michaelidis et al., 2005). Disturbances in acid-base balance are also known to place greater energy demands on marine organisms as homeostatic process becomes more energy intensive (Pörtner et al., 2004).

The acid-base balance of bivalves has been shown to be affected by elevated pCO_2 and trace metals interacting. Lewis et al. (2016) found that when the mussel *Mytilus edulis* was exposed to both elevated pCO_2 (1400 µatm) and copper (0.05 µM) there was a greater reduction in pH_e (Lewis et al., 2016). The authors hypothesised that mussels reduced their ventilation rates to minimise contact with the waterborne copper, and as a result experienced an accumulation of pCO_2 in their hemolymph, which in turn, decreased pH_e (Lewis et al., 2016). In contrast, this study found that elevated copper had no further effect on pH_e and elevated pCO_2 alone resulted in a reduction of pH_e. Oysters may not behave like mussels in response to waterborne copper. Previous studies have shown that aqueous copper concentrations must exceed 3200 µg L^{-1} before the oyster *Crassostrea gigas* lowers filtering rates (Lin et al., 1992).

After eight weeks of exposure, there were no significant effects of treatments on the SMR or ammonia excretion rate of adult oysters, but there was a significant effect of copper exposure on the O:N molar ratio of *S. glomerata*, with a trend for the combined copper and elevated pCO_2 treatment to have the greatest O:N ratio. The O:N molar ratio is an indicator of which catabolic substrate oysters use as energy (Bayne, 1999). A greater proportion of oxygen indicates more aerobic metabolism (using fats and carbohydrate) than protein catabolism (Wright,

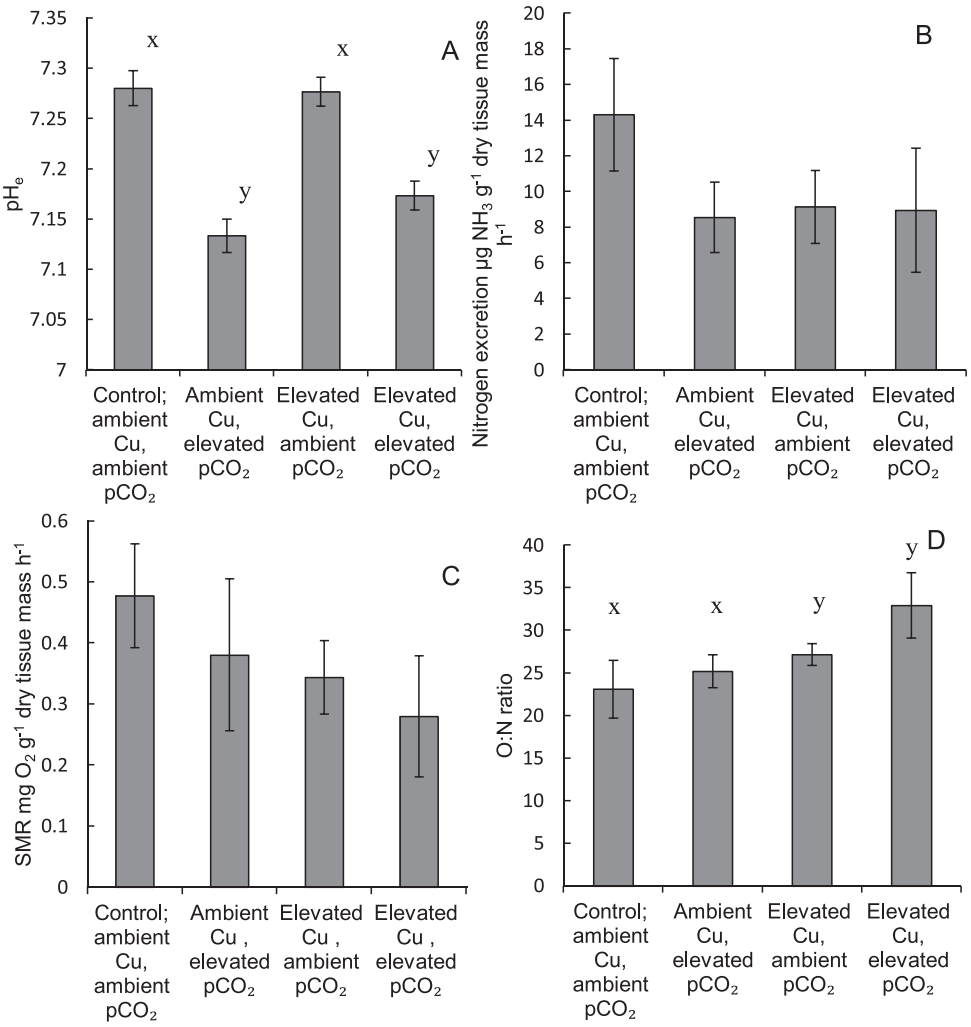

Fig. 2. Adult physiology. Mean (± SE) SMR O_2 mg g dry tissue mass^{-1} h^{-1} (n = 6; A),Nitrogen excretion NH_3 µg g dry tissue mass^{-1} h^{-1} (n = 6; B), pH_e (n = 3; C), O:N molar ratio (n = 6; C) of oysters following eight weeks exposure in experimental treatments. Letters (x–y) above columns indicate significant differences among treatments (SNK; $P < 0.05$).

Table 2
Mean [Cu] µg L^{-1} ± SE and Mean [Cu] µM ± SE (n = 3) measured in seawater per treatment using ICPMS in the 3rd and 7th week of experimental exposure.

		Ambient	Elevated pCO_2	Elevated Cu	Elevated pCO_2 + Cu
Week 3	Mean [Cu] µg L^{-1} ± SE	0.44 ± 0.04	0.54 ± 0.03	39.03 ± 2.98	38.49 ± 2.25
	Mean [Cu] µM ± SE	0.007 ± 0.0006	0.008 ± 0.0005	0.614 ± 0.04	0.606 ± 0.035
Week 7	Mean [Cu] µg L^{-1} ± SE	0.48 ± 0.06	0.47 ± 0.13	35.79 ± 1.3	36 ± 1.28
	Mean [Cu] µM ± SE	0.007 ± 0.0009	0.007 ± 0.002	0.563 ± 0.02	0.566 ± 0.02

1995). Oysters exposed to copper used proportionally more aerobic metabolism which is consistent with other studies that have found metabolic costs of metal exposure and increased protein synthesis required for cellular maintenance and repair (Sibly and Calow, 1989; Calow and Forbes, 1998; Sokolova and Lannig, 2008; Ivanina et al., 2008). The lack of observed physiological effects on SMR or nitrogen excretion may indicate that oysters have begun to acclimate to the experimental conditions and an energetic trade-off is occurring. Such a trade-off could be masking any whole organism responses (Sokolova, 2013). Previous studies that have exposed *S. glomerata* to similar levels of elevated pCO_2 for more than three weeks have found no significant effect on SMR (Parker et al., 2012; Scanes et al., 2017).

The copper content of *S. glomerata* exposed in the copper treatment were comparable with those measured in highly contaminated environments from eastern Australia (Edge et al., 2014), but the addition of elevated pCO_2 did not affect the copper content of oyster tissue. Previous work on metals and pCO_2 by Götze et al. (2014) found that the combination of elevated pCO_2 and copper synergistically increased the tissue load of [Cu] in both oysters (*C. virginica*) and clams (*M. mercenaria*) at a pCO_2 concentration of 800 µatm but had no effect at 1500 µatm. Other taxa such as cephalopods have shown different responses. Elevated pCO_2 exposure reduced the amount of accumulated cadmium (Cd) in the eggs of *Loligo vulgaris* and *Sepia officianalis* (Lacoue-Labarthe et al., 2009, 2011). Similarly, elevated pCO_2 reduced the amount of Cd accumulated in the tissues of the anemone *Anemonia viridis* (Horwitz et al., 2014). Increased respiration and SMR is often observed when bivalves and other invertebrates are exposed to elevated pCO_2 (Pörtner et al., 2004). By increasing respiration, bivalves expose themselves to a greater amount of dissolved trace metals. This is the mechanism that is often offered to explain why molluscs accumulate more trace metals

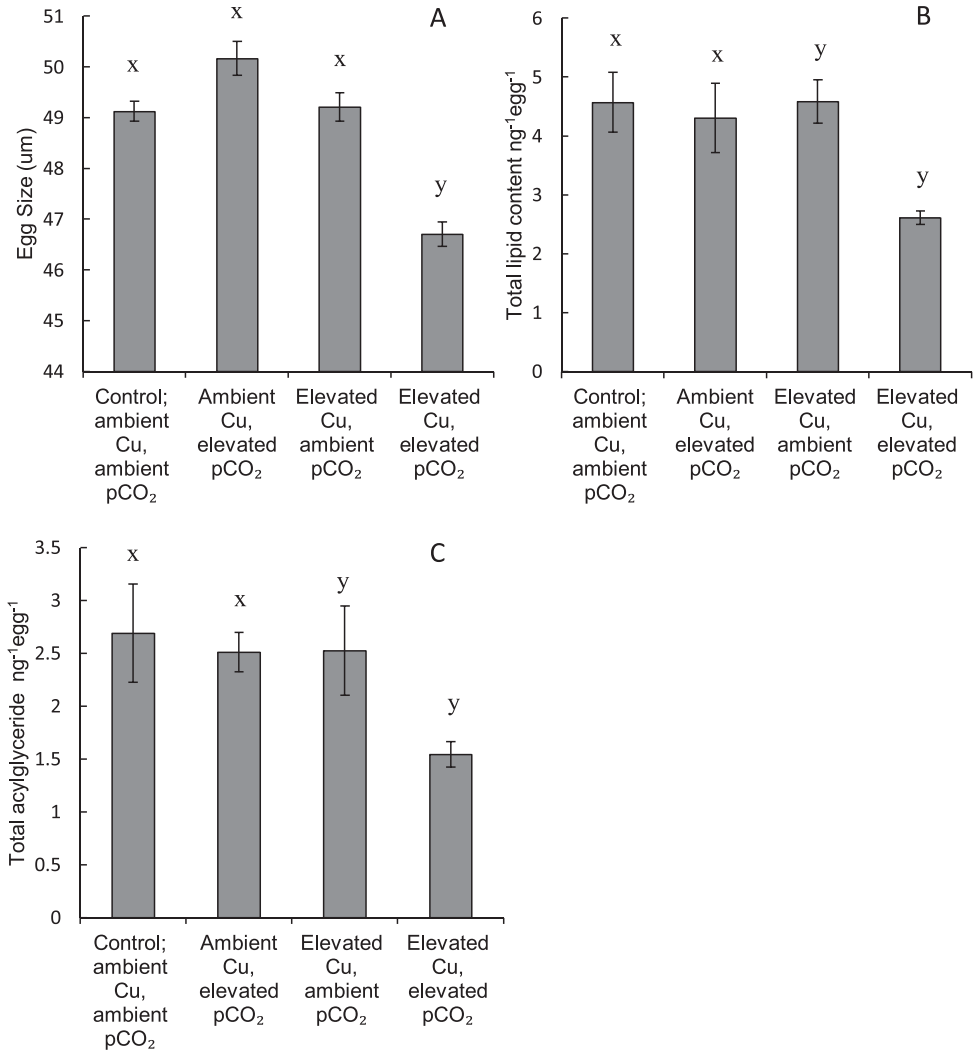

Fig. 3. Egg size and lipid content. Mean (± SE) egg size (n = 25 eggs; A), total lipid content ng^{-1} egg^{-1} (n = 3 tanks; B) and total acylglyceride content ng^{-1} egg^{-1} (n = 3 tanks; C) of eggs from female oysters following 8 weeks experimental exposure to treatments. Letters (x–y) above columns indicate significant differences (SNK; $P < 0.05$) among treatments.

under elevated pCO_2 (Ivanina et al., 2013; Götze et al., 2014). In this study, elevated pCO_2 had little effect on the SMR of *S. glomerata*, which may explain why there was no change in copper accumulation in soft tissue.

There were no strong interactive effects of elevated pCO_2 and copper on the metabolic rate of adult oysters in this study; but, the size of eggs and the total lipid and acylglyceride content was significantly reduced by the two stressors interacting. Energy resources may have been diverted towards homeostatic processes of adults and away from gamete production, resulting in lower egg energy content. A greater proportion of aerobic metabolism in copper exposed oysters (as indicated by O:N ratio) provides further evidence of lipid reserves being used for metabolism. The amount of energy that females invest in their gametes can indicate the energetic state of the females oysters themselves (Livnat et al., 2005; Marshall and Uller, 2007). Often, parental investment in invertebrates will be greatest when the mothers have a surplus of energy to expend on their offspring (Marshall and Keough, 2004; Marshall and Uller, 2007). These responses are not limited to invertebrates, terrestrial and marine vertebrates have also been shown to decrease the maternal investment of energy to their gametes when in contaminated environments (Alquezar et al., 2006; Verboven et al., 2009).

4.2. Offspring development

There was greater development to trochophores after 24 h in the combined elevated pCO_2 and copper treatment; which was the treatment with the lowest egg energy content. Trochophores are known to be a reliable indicator of larval development and reflect development success at later stages (Roberts, 1987; Matsuyama, 2003; Kurihara et al., 2009; Basti et al., 2011, 2013).

Marine organisms that are exposed to ocean acidification as parents can show positive carry-over effects, without apparent extra maternal energy investment (Miller et al., 2012; Donelson et al., 2012; Dupont et al., 2012; Parker et al., 2015, 2017a). In this study, the eggs with the lowest energy content had the greatest development to the trochophore stage. Previous work by Weng and Wang (2014) has shown that the tolerance of oyster (*Crassostrea sikamea*) offspring to trace metals is improved when their parents are also exposed. Weng and Wang (2014) found that the tolerant offspring expressed more proteins like metallothionein and this was likely responsible for their enhanced tolerance (although maternal investment was not measured; Weng and Wang, 2014). Furthermore, there is evidence that S. glomerata offspring are more tolerant to zinc if their parents are also exposed in polluted estuaries (Yingprasertchai et al., 2017).

Oysters in the elevated pCO_2 and copper treatment had smaller eggs,

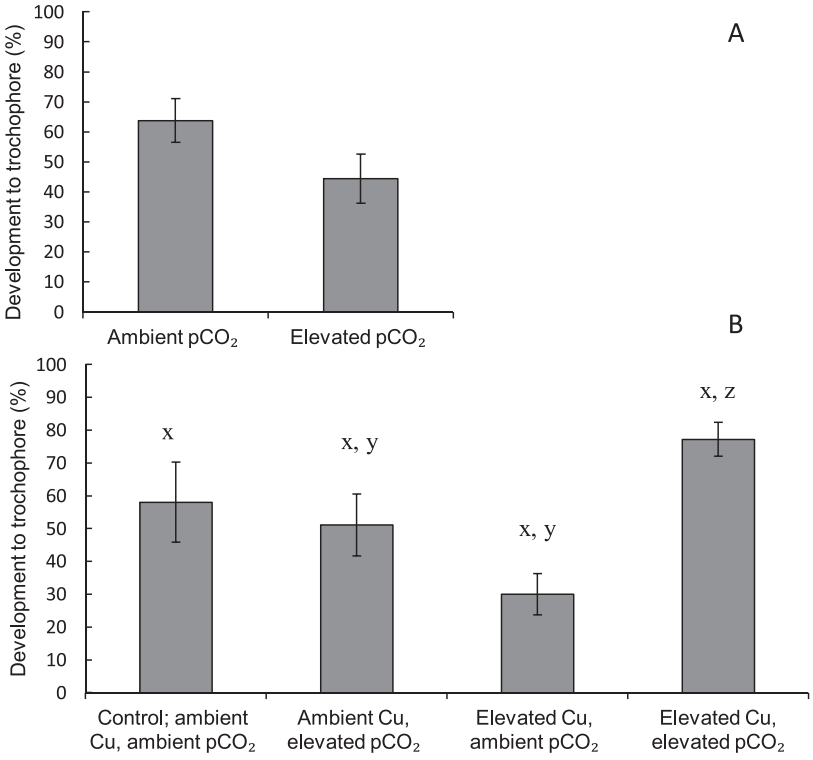

Fig. 4. Development of offspring. A) The mean (± SE) percentage development of embryos to the trochophore stage following parental exposure to experimental treatments (n = 12). B) The mean percentage development of embryos to the trochophore stage under either elevated or ambient pCO_2, irrespective of parental exposure to treatments (n = 24). Letters above bars represent results of SNK tests of this factor, corresponding letters (x–z) among bars indicate no significant difference (SNK; $P < 0.05$).

with a lower lipid and acylglyceride content. Acylglycerides are known to be the main source of endogenic energy for the embryos of marine broadcast spawning invertebrates (Yasumasu et al., 1984). Gallager et al. (1986) have shown a direct correlation between the total endogenous lipid content of eggs and the survival of those embryos in, *M. mercenaria,* and, *C. virginica*. Endogenous energy reserves of molluscan embryos must last the entire process of embryogenesis to the prodissoconch (D-veliger stage; Thorson, 1950) which is when mollusc larvae have the capacity to process exogenous energy. In this study, development was only measured until the trochophore stage. After the trochophore stage, there is still considerable development to be undertaken until the embryo reaches the veliger stage (when feeding can begin but cannot be solely relied upon) which will require further endogenous energy supplies (Morton et al., 1998). Here, energy may not have been a limiting factor in the development of offspring to the trochophore stage. However, if embryos were reared until veliger larvae, the lower energy content of eggs may have resulted in fewer veligers.

While copper exposure during embryonic development did not affect the rate of development, elevated pCO_2 reduced the development of offspring to trochophores. Elevated pCO_2 has been shown to significantly reduce fertilisation and development of trochophore and D-veliger larval stages including *S. glomerata* (Kurihara et al., 2009; Parker et al., 2009, 2010; 2013; Scanes et al., 2014). Sperm motility, a major contributor to fertilisation success, is also known to be reduced by elevated pCO_2 (Havenhand et al., 2008, although see Byrne et al. (2009) who found no effect on the urchin Heliocidaris erythrogramma). Early shell development that occurs during the trochophore stage is sensitive to the saturation state of aragonite ($\Omega_{aragonite}$) which is lowered by elevated pCO_2 (Waldbusser et al., 2015). Decreased sperm motility and decreased $\Omega_{aragonite}$ may explain the lower development of offspring under elevated pCO_2.

5. Conclusions

This study found that when *S. glomerata* were exposed to both copper and elevated pCO_2 during reproductive conditioning, the size and energy content of eggs were reduced but there was a positive carry-over effect on the development of offspring. There was little effect on physiology of adult oysters, elevated pCO_2 affected pH_e and Cu affected O:N ratios but the two stressors did not interact. These stressors did, however, interact to tower maternal investment. This suggests that the combined copper and pCO_2 treatment caused a greater energetic demand on oysters which was met by a metabolic trade-off, potentially allowing oysters to acclimate to stress. Such a finding was against predictions, but other studies have found that stressors can combine in unpredictable ways and when parents are exposed these interactions can influence the responses of their offspring. Our study highlights that oysters living in polluted environments such as urbanised estuaries may have some capacity to withstand ocean acidification. Future long term studies are required to determine the scale and persistence of long term positive or negative transgenerational carry-over effects.

Competing interests

The authors have no competing interests to declare.

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